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Long-term exposure to a continuous 900 MHz electromagnetic field disrupts cerebellar morphology in young adult male rats

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Abstract

The pathological effects of exposure to an electromagnetic field (EMF) during childhood and adolescence may be greater than those from exposure during adulthood. We investigated possible pathological changes in the cerebellum of adolescent rats exposed to 900 MHz EMF daily for 25 days. We used three groups of six 21-day-old male rats as follows: unexposed control group (Non-EG), sham-exposed group (Sham-EG) and an EMF-exposed group (EMF-EG). EMF-EG rats were exposed to EMF in an EMF cage for 1 h daily from postnatal days 21 through 46. Sham-EG rats were placed in the EMF cage for 1 h daily, but were not subjected to EMF. No procedures were performed on the Non-EG rats. The cerebellums of all animals were removed on postnatal day 47, sectioned and stained with cresyl violet for histopathological and stereological analyses. We found significantly fewer Purkinje cells in the EMF-EG group than in the Non-EG and Sham-EG groups. Histopathological evaluation revealed alteration of normal Purkinje cell arrangement and pathological changes including intense staining of neuron cytoplasm in the EMF-EG group. We found that exposure to continuous 900 MHz EMF for 1 h/day during adolescence can disrupt cerebellar morphology and reduce the number of Purkinje cells in adolescent rats.

Key words: adolescence, cell phone, cerebellum, electromagnetic field, Purkinje cells, rat

Cell phones have become an essential part of verbal and visual communication (Davis et al. 2012, 2013). Consequently, the possible pathological effects on humans of electromagnetic field (EMF) emitted by these devices currently are of great interest. Warnings about cell phone use, especially among children, have been issued in Australia, Belgium, France and other countries. Some countries have banned the sale of cell phones to children younger than 7 years and advertisements during children's TV programs or directed at children younger than 12 years also have been prohibited in some locations (Morgan et al. 2014).

The EMF emitted by cell phones can affect the functions and morphology of the brain, ears and eyes (Salford et al. 2003, Cardis et al. 2008). EMF appears to affect children and adolescents more than adults (Gandhi et al. 2012, Morgan et al. 2014). Gandhi et al. (1996) reported that electromagnetic waves penetrate more deeply into the brains of 5–10-year-old children than of adults. Some investigators have suggested that EMF emission by cell phones can reduce sperm number and quality, and alter motor behavior, particularly in children (Pourlis 2009, Sonmez et al. 2010,

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Hancı et al. 2013, Morgan et al. 2015). Others maintain that the EMF emitted by cell phones has no pathological effect on humans (Grafström et al. 2008, Corle et al. 2012, Sepehrimanesh and Davis 2016, Morgan et al. 2015).

The cerebellum is a suitable test organ for studies of EMF effects because disruption of cerebellar development for any reason results in uncoordinated and unbalanced movements (Sonmez et al. 2010). We used the stereological optical disector method to estimate total numbers of Purkinje cells (Geuna 2005, Bas et al. 2009a,b, İkinci et al. 2013). We used 900 MHz EMF because the majority of cell phones in the Global System for Mobile telecommunications (GSM) operate at this frequency (Dubreuil et al. 2002, 2003).

Adolescence in rats is defined as postnatal days (PD) 21–60. This period can be subdivided further into early adolescence (PD 21–34), mid-adolescence (PD 34–46) and late adolescence (young adulthood) (PD 46–59) (Tirelli et al. 2003). We investigated whether the cerebellum of young adult rats was affected by exposure to continuous 900 MHz EMF for 1 h/day for 25 days during early and middle adolescence using morphological and stereological techniques.

Material and methods

Animals and animal care

Experiments and surgical procedures were performed in the Karadeniz Technical University (KTU) Experiment Animals Surgical Research and Application Center (Trabzon, Turkey) (KTU-EXAC) and in the KTU Medical Faculty Department of Histology and Embryology. All experimental procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council (US) Committee Update of the Guide for the Care and Use of Laboratory Animals 2011). Our animal study protocols and experimental procedures were approved by the KTU Animal Care and Ethics Committee as required by law for animal experiments in Turkey.

Eighteen 21-day-old male Sprague-Dawley rats were purchased from the KTU-EXAC and housed in standard laboratory cages in the KTU-EXAC. During the experiment, all cages containing rats in the experimental groups were located in a resting room in the KTU-EXAC away from the EMF generator during EMF and sham procedures. The resting room was equipped with a HEPA filtered air supply. The temperature and relative humidity were maintained at 22 ± 2° C and 50 ± 10%, respectively, and a 12 h light:12 h dark cycle (lights on 07:00–19:00) was used. All rats were allowed unrestricted access to water and a commercial diet (Bayramoğlu Feed and Flour Industry Trading Corp., Erzurum, Turkey). A separate exposure room within the KTU-EXAC, with conditions identical to those in the resting room, was used only for EMF and sham procedures. Rats in the sham or EMF treatment groups were moved to the exposure room for their respective procedures, then returned to the resting room.

The application system has been described in detail elsewhere (İkinci et al. 2013, Odacı et al. 2013, 2016). The EMF cage was made of Plexiglas and was 42 × 30 × 50 cm high. EMF was generated using an ultra-high frequency oscillator (1218-BV; lockable oscillator, 900–2000 MHz, General Radio Co., Concord, MA) with an uninterrupted power source (1267-B; regulated power supply, General Radio Co.) with an output power of approximately 300 mW and the frequency adjusted to 900 MHz. The oscillator was attached by a coaxial cable to a copper rod (1 mm × 15 cm) half-wave dipole antenna. The antenna was fixed in the center of the EMF cage. An insulating wooden rod was used to fix the antenna in the center of the EMF cage. The antenna was attached to the center of the rod, and installed equidistant from the right and left sides and approximately 11 cm within the opening of the EMF cage (Fig. 1).

Experimental design

Rats were divided randomly into three groups of six animals. Group 1 was the unexposed control group (Non-EG). No procedure was performed on the rats in this group; they were maintained in their own cages throughout the study. Group 2 was the sham-exposed group (Sham-EG). Rats in this group were transferred to the EMF cage for 1 h/day for 25 days (PD 21–46) at the same time each day (09:00–10:00), but were not exposed to EMF. The Sham-EG rats subsequently were returned to the resting room. Group 3 was the EMF-exposed group (EMF-EG). These animals were placed in the EMF cage and exposed to 900 MHz EMF for 1 h/day for 25 days (PD 21–46) at the same time each day (10:00–11:00). EMF-EG rats were returned to the resting room after exposure to EMF. The three groups of rats were kept in three separate cages in the resting room.

EMF intensity and temperature

The possibility of unintended EMF effects in the exposure room was assessed using a broad band

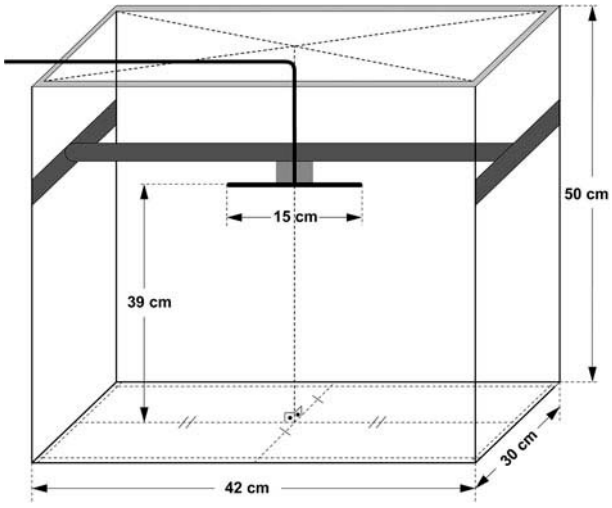

Fig. 1. Schematic of the EMF cage and the location of the 15 cm copper dipole antenna. The antenna was fixed in the center of the EMF cage using an insulating wooden rod and was used to apply the EMF transmitted from the oscillator by a coaxial cable.

electric field measuring device (C.A 43 isotropic electrical field intensity meter; Chauvin Arnoux Group, Paris, France) with a 100 kHz–2.5 GHz measurement range. The daily EMF and sham applications were performed only after the absence of other EMF inside the exposure room had been confirmed. Rats were allowed to move around freely inside the EMF cage during the EMF and sham periods to minimize stress from cage changes and immobility. In addition, the intensity of EMF was measured every day on the floor of the EMF cage to determine the distribution of EMF intensity in the area that contained the rats. Measurements were made with rats inside the EMF cage, with the EMF cage empty, and from the floor of the EMF cage and beneath it using the magnetic field measuring device described above. From these data, the whole body specific absorption rate (SAR) was calculated using the RadHaz SAR equivalence calculator, Version 1.0 (Richard Tell Assoc., Inc., Mesquite, NV) following the procedures of İkinci et al. (2013) and Odacı et al. (2013). The temperature within the EMF cage during both EMF and sham periods was measured and recorded each day for 25 days using a digital thermometer (YCOM KMN-303, YuYau Shuanghe Electron Instrument Co. Ltd., Zhejing, China).

Tissue preparation

All rats were weighed and sacrificed at the end of the experiment (PD 47) by decapitation under deep anesthesia using 50 mg/kg Ketalar (Eczacıbaşı, Istanbul, Turkey). The cerebellum of each animal was removed under sterile conditions, fixed in 10% neutral buffered formalin for 10 days, processed routinely through graded alcohols and xylene, and embedded in paraffin. Coronal 30 µm serial sections were cut using a fully automatic microtome (RM 2255; Leica Instruments, Nussloch, Germany). Each section was placed on a slide coated with gelatin-formaldehyde. Sections were deparaffinized in xylene and re-hydrated through a graded alcohol series. Cresyl violet staining was performed for histopathological and stereological analyses (Bancroft et al. 1996). We examined the sections using an Olympus BX51 light microscope (Olympus, Tokyo, Japan). Images were obtained using a camera microscope (DP 71; Olympus).

Optical disector technique

The number of Purkinje cells in the sections of cerebellum was determined using the optical disector technique as described earlier (Gundersen and Jensen 1987, Tunc et al. 2007). For this purpose we used a stereological application system controlled by stereology software and a personal computer running stereology analysis software (Stereo Investigator 9, computer-assisted stereological toolbox; MicroBrightField Bioscience, Williston, ND) designed for stereological studies. Other equipment included a research microscope (DM4000B-M; Leica, Stuttgart, Germany), a charge coupled device camera (JVC, Yokohama, Japan), a measuring rod operating on the Z axis (LIP 401 R, Heidenhain, Traunreut, Germany), a microcator indicator (ND 221 B; Heidenhain) and a computer controlled step motor whose movements along the X and Y axes were controlled by a PC and a joystick (Prior Scientific Inc., Rockland, MA). Purkinje cell counting was performed by one of the co-authors blinded to the identity of the group.

We conducted a pilot study to optimize the sampling and counting strategy for stereological calculations. Eighteen or 19 slides from each cerebellum are sufficient for estimating total neuron numbers for rats using the optical disector method (Sonmez et al. 2010). We selected randomly every seventh slide from the series of slides to be analyzed. Once the first slide had been selected at the beginning of the slide series for analysis, sampling then commenced from a random section between one and seven. The efficacy of the sampling strategy and the number of sampled cells for calculating total neuron numbers were validated using coefficient of error

(CE) and coefficient of variation (CV) values from previous stereological reports (Gundersen and Jensen 1987, Bas et al. 2009b, Odaci et al. 2008, 2010, West et al. 1991). In this way, we confirmed sampling reliability and the appropriate number of sampled cells for calculating total neuron numbers (Gundersen and Jensen 1987). The CE should be ≤10% (Gundersen and Jensen 1987, West et al. 1991). It also was possible to estimate the CV for the cerebellum in each group to confirm that the number of subjects in each group was sufficient. The counting procedures employed mean CV for each group, mean CE for stereological estimation of Purkinje cell numbers and other stereological parameters are presented in Table 1.

We counted the number of Purkinje cells in the cerebellum of all animals as described earlier (Bas et al. 2009b). Briefly, if the largest nuclear profile came into focus within the unbiased virtual counting frames spaced randomly and systematically throughout the delineated regions, then the Purkinje cell was counted. Total Purkinje cell numbers were calculated based on the number of Purkinje cells counted and the sampling probability (Bas et al. 2009b, West et al. 1991). All details of the counting and stereological procedures are summarized in Table 1.

Statistical analysis

All statistical analyses were performed using SPSS software (version 11.0, SSPS Inc., Chicago, IL). The numbers of Purkinje cells are expressed as means ± SD for all groups. Values among the groups were analyzed using one-way analysis of variance (ANOVA) based on the Levene and Shapiro Wilk test results for equality of variances and normality assumptions, respectively. Tukey's HSD multiple comparison test was applied to determine additional differences between groups. Significance was set at $p \leq 0.05$ for all tests.

Results

EMF levels

Based on measurements taken every day throughout the study with the EMF cage empty, the mean EMF intensity was 6.8 V/m beneath the EMF cage and 11.9 V/m within it. The EMF values with rats inside the EMF cage were 4.5 V/m beneath the EMF cage and 13.4 V/m within it. The whole body specific absorption rate (SAR) was 0.01 W/kg.

Temperature within EMF cage

The mean temperature within the EMF cage was 21.4 ± 1.7° C during exposure to EMF and 21.7 ± 1.7° C during the sham period. The difference was not statistically significant.

Numbers of Purkinje cells

The number of Purkinje cells in the EMF-EG group was significantly less than for the Non-EG and Sham-EG cell groups ($p < 0.001$) (Table 1). Differences in numbers of Purkinje cells between the Non-EG and Sham-EG groups were not statistically significant.

Table 1. Total Purkinje cell numbers and stereological analysis data for estimation of total Purkinje cell numbers in the rat cerebellums in the study groups

	Non-EG	Sham-EG	EMF-EG
Total Purkinje cell number	317.32 ± 16	332.26 ± 57.00	257.46 ± 10[a]
Disector particle number	236.20 ± 16.81	248.00 ± 12.52	191.00 ± 16.48
Number of sampled sections	19.80 ± 3.06	19.50 ± 2.35	18.00 ± 1.79
Average section thickness (μm)	21.10 ± 0.91	22.00 ± 1.44	21.40 ± 1.44
Number of steps for counting	149.00 ± 5.90	157.70 ± 3.14	119.00 ± 5.33
Section sampling fraction (ssf) (coronal)	1/7	1/7	1/7
Counting frame size (μm^2)	598	598	598
Area sampling fraction (asf) ($\mu m^2/\mu m^2$)	598/90000	598/90000	598/90000
Thickness sampling fraction (tsf) (μm/μm)	10/21.1	10/22	10/21.4
CE (mean)	0.06	0.06	0.06
CV	0.03	0.03	0.03

Data are means ± SD; $n = 6$ for each group. Non-EG, Non-exposed group; Sham-EG, sham-exposed group; EMF-EG, EMF-exposed group; SD, standard deviation; EMF, electromagnetic field; CE, coefficient of error; CV, coefficient of variation. [a]Numbers of Purkinje cells in the EMF-EG group decreased compared to the Non-EG and Sham-EG groups ($p < 0.001$).

Histopathology

Histopathological evaluations of sections from Non-EG (Fig. 2A), Sham-EG (Fig. 2B) and EMF-EG (Fig. 2C) groups were made using light microscopy. Normal cerebellar morphology and normal cytoplasm were observed in Purkinje and other cerebellar neurons at all magnifications in the Non-EG and Sham-EG sections. We observed alteration of normal Purkinje cell arrangement and pathological changes, such as more intense staining of neuron cytoplasm in the EMF-EG sections (Fig. 2C).

Discussion

We found that the number of Purkinje cells in EMF-EG rats was significantly lower than both the Non-EG and Sham-EG groups. Our finding is consistent with previous investigations of rats at various ages. Sonmez et al. (2010) investigated the effects of exposure to 900 MHz EMF for 1 h/day for 28 days on the number of cerebellar Purkinje cells in 16-week-old female rats. These investigators reported that the number of Purkinje cells for the EMF group was significantly lower than for the Non-EG and Sham-EG groups. We used an optical disector technique to estimate cell numbers, whereas an optical fractionator technique was used for the earlier study. Both techniques are stereological methods and are effective, unbiased ways to calculate cell numbers (Sterio 1984, Geuna 2005, Bas et al. 2009a,b, İkinci et al. 2013). Rağbetli et al. (2010) investigated the effect of cell phones on the numbers of Purkinje and granule neurons in the developing cerebellum using the optical disector method and reported a significant decrease in the number of Purkinje cells and a tendency toward increased numbers of granule cells.

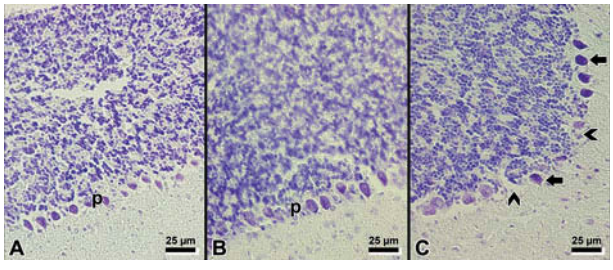

Fig. 2. Sections of rat cerebellum from Non-EG (A), Sham-EG (B) and EMF-EG (C) rats. Non-EG and Sham-EG sections exhibited normal cerebellar morphology, typical Purkinje cell arrangement and normal Purkinje cell cytoplasm (p). Altered Purkinje cell arrangement (angled arrow) and more intense staining of neuron cytoplasm (arrow), can be seen in EMF-EG sections. H & E. All panels × 400.

Physical examination of EMF-EG rats revealed no gross anatomical anomalies and we found no significant differences body weights among the groups at the end of the experiment. These findings suggest that 900 MHz EMF did not cause gross anatomical alterations or changes in body weights and were free from stress-related effects during EMF exposure. We observed alteration of normal Purkinje cell arrangement and more intense staining of neuron cytoplasm in EMF-EG sections. These findings together with the decreased number of Purkinje cells, suggest that long term and continuous exposure to 900 MHz EMF during early and middle adolescence causes pathological effects on the young adult rat brain at the cellular level. Consistent with our findings, Salford et al. (2003) reported dark neurons interspersed among the normal hippocampal pyramidal cell band in rats exposed to EMF. The cerebellum has been shown to be particularly susceptible to exposure to 900 MHz EMF (Sonmez et al. 2010).

The abnormal Purkinje cell arrangement and intensely stained cytoplasm in neurons of EMF-EG rats may be due to neuronal injury associated with oxidative stress induced by 900 MHz EMF. Kerimoğlu et al. (2016) investigated 60-day-old male rat hippocampus following exposure to 900 MHz EMF and reported increased numbers of pyknotic neurons with black or dark blue cytoplasm and fewer pyramidal neurons in the EMF-exposed group. These investigators also reported increased malondialdehyde and glutathione levels, but decreased catalase levels in EMF-exposed animals. These investigators concluded that their findings indicated oxidative stress-related morphological damage and pyramidal neuron loss in rat hippocampus following exposure to 900 MHz EMF during the adolescent period. Our findings are consistent with previous reports and we surmise that the pathological changes in Purkinje cells in male rats exposed to 900 MHz EMF are be attributable to oxidative stress.

Our findings indicate that long-term and continuous exposure of rats to 900 MHz EMF during early and middle adolescence can produce pathological effects including a decreased number of Purkinje cells. Further studies of acute and chronic exposure to a range of EMF for various periods are required for comparison with our results.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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